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13. ABSTRACT (Maximum 200 Words) Activation of calpains and caspases are major pathological events in traumatic brain injury (TBI). This proposal addresses four hypotheses: (1) TBI produces early and sustained increases in calpain and caspae-3 activity that vary depending upon the brain regions studies. (2) TBI can produce differential activation of calpain isoforms (μ -calpain, m-calpain) in different regions, and changes in calpain activity may be differentially localized in subcellular fractions (cytosolic vs. membrane). (3) TBI produces changes in calpastatin mRNA and differential expression of calpastatin isoforms in different brain regions. Changes may be differentially localized in subcellular fractions. (4) Increased magnitudes and duration of calpain and caspae-3 activation following TBI are predictive of morphopathology including focal contusion as well as necrotic and apoptotic cell death. Morphopathological changes, including apoptotic and necrotic cell death, can occur in regions showing activation of calpain but not caspase 3-like proteases. Recent technical advances have enhanced opportunities to study mechanisms of calpain and caspse-3 proteolysis in TBI, and the proposed research will refine powerful new methods.				
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INTRODUCTION

A large body of research has focused on the pathological significance of calcium accumulation in the central nervous system (CNS) following cerebral ischemia, spinal cord injury and traumatic brain injury (TBI). Disturbances in neuronal calcium homeostasis may result in activation of several calcium sensitive enzymes including lipases, kinases, phosphatases and proteases. Recent studies have provided substantial evidence that activation of the calpains, calcium activated intracellular proteases, is a major pathological event in a number of acute CNS insults including TBI. Similarly, recent data have implicated another important family of cysteine proteases, caspases – especially caspase-3.

Much of the evidence on calpain and caspase activation following TBI has been indirect. The current project incorporates an integrated approach to studying protease activity in a clinically relevant rodent model of cortical impact injury using complementary techniques. Western blot immunoassays of calpain and caspase-3 specific BDP's proposed in SOW1 will be an efficient means of determining which brain regions and time points may be most usefully incorporated into studies in SOW2 providing more detailed information on mechanisms of calpain activation. Once we have a thorough understanding of calpain activity following TBI, we will initiate studies of calpastatin in SOW3. SOW4 will then investigate predictive relationships between changes in calpain and caspase activity and features of morphopathology produced by TBI. Studies will initially focus on five brain regions (cortex, dorsal hippocampus, thalamus, striatum and corpus callosum) taken from zones ipsilateral and contralateral to the site of cortical impact injury.

BODY

Record Of Research Findings and Approaches

As outlined in the proposal, during the first year of research, Dr. Hayes moved his laboratory from the University of Texas-Houston Medical School to the Evelyn F. and William L. McKnight Brain Institute of the University of Florida. As outlined in the approved statement of work, year 1 studies focus on testing the hypothesis that TBI produces early and sustained increases in calpain and caspase-3 activity, inferred by increases in calpain and caspase-3-specific breakdown products (BDPs), that vary depending upon the brain region studied. Increases in calpain activity can occur independently of caspase-3 activation. The studies examine tissue extracts from selected brain regions for evidence of calpain activation at various times after TBI by measuring changes in levels of calpain-specific BDPs (145 kDa) and caspase-3-specific BDPs (120 kDa) of α -spectrin using semi-quantitative Western immunoassays. Studies of calpain and caspase-3-specific BDPs of α -spectrin include preparation and characterization of fragment-specific antibodies. Caspase-3 (and caspase-1) activation will ultimately be examined by measuring fluorogenic substrate cleavage.

In spite of the laboratory move, we have made considerable progress in Western blot characterization of calpain and caspase-3 activity following TBI. These studies have provided the exciting and novel observation that calpain activation is considerably more prolonged than previous studies in our laboratory had suggested (Pike et al., 1998).

Results

Calpain-specific α -spectrin fragmentation (145 kDa) was detected at the earliest time-point assayed (3h) in all brain regions except striatum after 1.6 or 2.0 mm TBI. The 145 kDa fragment was no longer detectable in cortex and hippocampus by 14 days post-injury. Interestingly, the 145 kDa fragment was still detectable in thalamus after 1.6 mm, but not 2.0 mm injury at 28 days post-injury. The pattern of calpain activation closely paralleled the pattern of neuronal atrophy in these regions as assayed by H&E staining. Increases in the caspase-3 specific fragment to α -spectrin (120 kDa) was not detected at any post-injury time point (See Figure in Appendix).

Materials and Methods

Surgical preparation and controlled cortical impact brain injury: Forty adult male Sprague-Dawley rats were anesthetized with 4% isoflurane in a carrier gas of 70% N₂O/30% O₂, intubated and mechanically ventilated on 2% isoflurane in 70% N₂O/30% O₂. Core body temperature was monitored continuously by a rectal thermistor probe and maintained at 36.5-37.5 degrees C. A unilateral craniotomy (7 mm diameter) was performed over the right cortex adjacent to the sagittal suture, midway between bregma and lambda. Care was taken to avoid penetrating the underlying dura matter.

Trauma was produced by impacting the exposed right cortex with a 6 mm diameter aluminum tip at a velocity of 4 m/s with a 2.3 mm compression. Sham-injured animals underwent identical surgical procedures but were not subjected to impact injury. Following injury or sham-injury, animals were weaned from the ventilator and extubated. Appropriate pre- and post-injury management was maintained to insure that all guidelines set forth by the Guide for the Care and Use of Laboratory Animals were complied with.

Immunoblot analysis: Ten groups (n = 4 per group) of rats were used in this study. Groups included sham-injured animals and traumatized animals sacrificed at post-injury time points of 15 min., 3, 6, 24, 48 and 72 hours and 7 and 14 days. At appropriate post-injury time points, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and decapitated at the loss of the toe-pinch reflex. Following decapitation, ipsilateral and contralateral (to the site of impact) cortex, hippocampus, thalamus, and striatum were rapidly dissected, quickly frozen in liquid N₂, and stored at -80 degrees C.

Brain tissue was homogenized in ice cold homogenization buffer (20 mM PIPES pH 7.6, 2mM EGTA, 1mM EDTA, 1mM DTT, 0.5 mM PMSF, 50 μ g/ml each of AEBSF, aprotinin, pepstatin., TLCK and TPCK). Protein concentrations were determined by bicinchoninic acid micro protein assays (Pierce, Inc., Rockford, IL) with albumin standards. Protein-balanced samples were prepared for polyacrylamide gel electrophoresis (PAGE) in a two-fold loading buffer (0.25 M Tris pH 6/8, 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H₂O), heated for 10 min at 95 degrees C, and centrifuged for 1 min at 10,000 r.p.m. Thirty micrograms of each sample was resolved in a vertical electrophoresis chamber using 4% acrylamide stacking gel over a 7% acrylamide resolving gel for 1 hour at 200 V. For immunoblotting, separated proteins were laterally transferred to nitro-cellulose membranes using a transfer buffer consisting of 0.192 M glycine and 0.025 M Tris (pH 8.3) with 10% methanol at a constant voltage of 100 V for 1 hour at 4 degrees C. Blots were blocked overnight

in 5% non-fat milk in 20 mM Tris-HCl, 0.15 M NaCl, and 0.005% Tween-20 at 4 degrees C. Comossie blue and Panceau red (Sigma, St. Louis, MO) were used to stain gels and nitrocellulose membranes (respectively) to confirm that equal amounts of protein were loaded in each lane.

Antibodies and immunolabeling: Immunoblots were probed with an anti-spectrin monoclonal antibody (clone AA6; catalog # FG 6090; Affiniti Research Products Ltd., UK) that detects intact non erythroid α -spectrin (240 kDa) and 150, 145 and 120 kDa α -spectrin breakdown products (SBDPs). The 150 kDa SBDP has been reported to be an α -spectrin cleavage product of calpains and caspase-3-like proteases. However, the 145 kDa SBDP is a specific proteolytic fragment of calpains, whereas the 120 kDa SBDP is a specific proteolytic fragment generated by caspase-3 (CPP32) activation. Following incubation with the primary antibody (1:6000 dilution) for 2 hours at room temperature, nitrocellulose membranes were incubated with a secondary antibody linked to horseradish peroxidase (dilution 1:20 000) for 1 hour. Enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) reagents were used to visualize immunolabeling on high performance chemiluminescence film (Hyperfilm-ECL; Amersham International, UK).

KEY RESEARCH ACCOMPLISHMENTS

- Completion of series of experiments characterizing prolonged calpain activation (in the absence of significant caspase-3 activation) following TBI *in vivo*.
- Preparation and *in vitro* characterization of antibody specific to activated mu- and mili- calpain, important components of research in SOW2, year 2.
- Initiation of fluorometric assays to characterize calpain and caspase-3 activation.
- Move of laboratory from University of Texas-Houston Medical School to the University of Florida.

REPORTABLE OUTCOMES

R.L. Hayes, X. Zhao, J.K. Newcomb, C.G. Markgraf and B.R. Pike. Calpain is activated for up to one month in the thalamus after moderate or severe traumatic brain injury. Abstract for National Neurotrauma Society 1999.

B.R. Pike, E. Johnson, J. Flint, C.C. Glenn, R.L. Hayes. Prolonged calpain activation in regions of tissue atrophy after traumatic brain injury. Abstract for International Neurotrauma Society 2000.

CONCLUSIONS

The research within the first year has provided novel and exciting information indicating that calpain activation following TBI occurs for considerably longer periods (up to 28 days) than previously thought. These data have a number of important scientific and clinical implications.

First, prolonged calpain activation may either be injurious, producing destruction of important structural cytoskeletal proteins, or an important component of repair mechanisms critical to recovery of function. The pathological or restorative functional significance of calpain activation will be directly investigated in SOW4. These studies also have critical clinical implications. There is currently no effective treatment for TBI, and a large number of recent clinical trials have proven negative. However, most clinical trials attempt to treat TBI injury for only the first few days after injury. Our data suggests that potentially injurious calpain activation could extend for far longer periods than previously recognized. Thus, therapies blunting destructive proteolysis by calpain may need to be maintained for far longer than previously thought. The current studies, and additional studies incorporated in this project, will provide information essential to the appropriate design of therapies to treat TBI in humans.

REFERENCES

Brian R. Pike, Xiurong Zhao, Jennifer K. Newcomb, Rand M. Posmantur, Kevin K.W. Wang and **Ronald L. Hayes**. Regional calpain and caspase-3 proteolysis of α -spectrin after traumatic brain injury. NeuroReport 9: 2437-2442, 1998.

APPENDICES

One Figure and Figure Legend is attached.

FIGURE

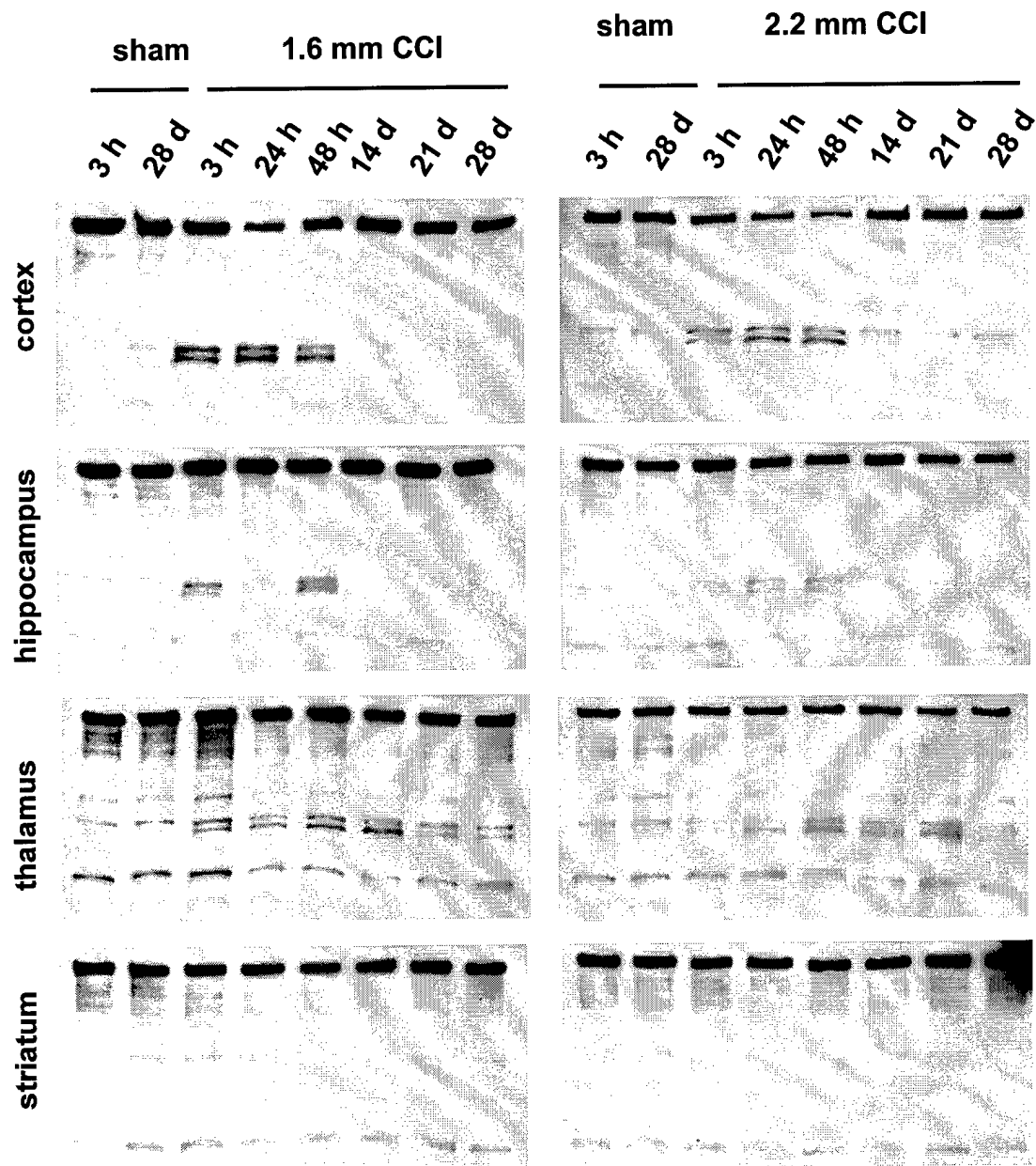


FIGURE LEGEND

Traumatic brain injury results in regional and temporal differences in calpain and/or caspase-3 protease cleavage of α -spectrin in brain regions ipsilateral to the site of impact. Cortex: primarily a calpain-mediated event characterized by rapid proteolysis of α -spectrin by calpain to a 145 kDa fragment by 3 h reaching maximal accumulation by 48 h post-injury. No increases in the 120 kDa fragment by caspase-3 were detected. Hippocampus: rapid but brief proteolysis of α -spectrin to a 120 kDa fragment by caspase-3 from 15 min to 6 h post-injury followed by delayed but sustained calpain activation indicated by accumulation of the 145 kDa fragment from 24 h to 7 days post-injury. Thalamus: rapid and increasing accumulation of calpain-specific 145 kDa fragment from 15 min to 28 days post-injury with brief accumulation of the 120 kDa caspase-3 fragment between 15 min and 3 h post-injury. Striatum: primarily a caspase-3 mediated event characterized by accumulation of 120 kDa fragment from 3 h to 48 h with minimal detectable levels of the calpain-specific 145 kDa fragment.